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Comparison of traditional intranasal and aerosol inhalation inoculation of mice with influenza A viruses

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Abstract

Intranasal instillation of virus in a liquid suspension (IN) is the most frequently employed method to inoculate small mammalian models with influenza virus, but does not reflect a natural route of exposure. In contrast, inoculation via aerosol inhalation (AR) more closely resembles human exposure to influenza virus. Studies in mice have yielded conflicting results regarding virulence induced by virus inoculated by these routes, and have not controlled for potential strain-specific differences, or examined contemporary influenza viruses and avian viruses with pandemic potential. We used a whole-body AR inoculation method to compare infectivity and disease progression of a highly pathogenic H5N1, a low pathogenic H7N9, and a 2009 H1N1 virus with traditional IN inoculation in the mouse model. Generally comparable levels of morbidity and mortality were observed with all viruses examined using either inoculation route, indicating that both IN and AR delivery are appropriate for murine studies investigating influenza virus pathogenicity.

Keywords

Influenza; Animal model; Aerosol; Infectivity

Introduction

The use of small mammalian models is critical for the study of both influenza virus pathogenicity and the assessment of interventions to mitigate disease severity. While numerous laboratory species have been established to characterize the pandemic potential of influenza viruses, including the ferret, guinea pig, and non-human primate, mice remain the most prevalent model due to their relatively lost cost, ease of handling, and wide array of available reagents (Belser et al., 2009). Intranasal instillation with a liquid inoculum (IN) remains a standard method of virus inoculation in all laboratory species. However, previous work has shown that the route and volume of virus administered to mice, or the anesthetic used during inoculation, can greatly influence the resulting morbidity and mortality following infection, especially when studying highly pathogenic avian influenza (HPAI) viruses (Belser and Tumpey, 2013; Knight et al., 1983; Miller et al., 2013). As inhalation of

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virus-containing aerosols represents a more natural route of human exposure compared with liquid instillation, several aerosol inhalation (AR) methods for inoculation with influenza virus have been established (Gustin et al., 2012; Schulman and Kilbourne, 1963; Snyder et al., 1986). These studies have revealed that aerosol inhalation can lead to a productive virus infection in numerous mammalian species, resulting in equivalent or enhanced severity of disease compared with traditional liquid instillation of virus.

Mice represent the most frequently utilized model for evaluating the virulence of influenza viruses following IN or AR administration. Several experimental protocols have been established to inoculate mice by the AR route, delivering virus-containing aerosols to mice via nose-only inhalation systems which require the use of anesthesia, restraint tubes, and/or training of animals prior to exposure, or whole-body exposure systems without anesthesia (Bowen et al., 2012; Johansson and Kilbourne, 1991; Larson et al., 1976; Sherwood et al., 1988; Smith et al., 2011). Conclusions drawn from these studies are conflicting; some studies demonstrated enhanced virulence and infectivity following AR inoculation compared with IN (Bowen et al., 2012; Smith et al., 2011), whereas others observed enhanced lethality following IN compared with AR inoculation (Johansson and Kilbourne, 1991), or comparable results by either inoculation method (Frankova, 1975; Larson et al., 1976; Sherwood et al., 1988). Variability in inoculation procedures, inoculating dose and volume, mouse age, virus strain, and size of generated aerosols all likely contribute to this disparity. Furthermore, all of these studies were performed with H1N1 and H3N2 strains (wild-type or lab-adapted), limiting our ability to apply these findings to current viruses of concern, notably HPAI and low pathogenicity avian influenza (LPAI) strains. There is a need to assess if existing models that utilize IN inoculation are not over or under-estimating the infectivity or virulence of viruses with pandemic potential compared with AR delivery. Here, we established a whole-body AR model to inoculate mice with three influenza A viruses which exhibit differential virulence in the mouse model and compared infectivity, disease progression, and viral titers with traditional IN inoculation (Table 1).

Numerous studies have found that aerosol particles <1 μm represent the dominant size of expelled aerosols in the exhaled breath of humans (Fabian et al., 2008; Papineni and Rosenthal, 1997). To simulate this exposure, murine AR inoculations were performed using equipment and experimental conditions previously described using a Collison nebulizer to generate aerosols in this range (Gustin et al., 2011). Unanesthetized female BALB/c mice (Jackson Laboratories) were placed inside a whole-body aerosol exposure chamber in animal holding cages (9″×4.5″×5″) (Biaera) for the duration of a 15-min exposure while the animals were exposed to aerosolized virus. Following removal of mice from holding cages, surface decontamination of mouse fur was performed by wiping the animals with isopropanol wipes. To estimate the amount of virus inhaled by mice during the inoculation period (the “presented dose”), we measured the concentration of virus generated in the exposure chamber and multiplied that by the exposure time and respiratory minute volume of mice (0.06 L/min) as described previously (Flandre et al., 2003; Gustin et al., 2011). Because each virus stock responds differently to the aerosolization process, prior to animal experiments, we established the starting virus concentration required in the nebulizer to achieve a particular virus concentration in the exposure chamber (within one log). This procedure was repeated until the dosimetry of each virus stock was well established. Based

on these results, reproducible aerosol inoculations of animals are made possible. Therefore, unlike traditional IN inoculation, where serial 10-fold dilutions of liquid inoculum are based on virus titers alone, aerosol inhalation inoculations are based on virus titers, respiration, time, and the sensitivity of each virus to the aerosolization process, demonstrating the reproducibility of viral titers achieved in the exposure chamber as serial dilutions of the initial virus titers in the nebulizer which correspond with a serially diluted presented dose. All animal research was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility, under the guidance of the CDC's Institutional Animal Care and Use Committee.

To determine if a HPAI virus maintained comparable lethality following AR inoculation, mice were inoculated by the AR or IN route with serial 10-fold dilutions of A/ck/Korea/Gimje/08 (Gimje/08), a HPAI H5N1 virus which exhibits a lethal phenotype in mice following IN inoculation (Belser et al., 2013b). All IN inoculations were performed with 50 μl of diluted virus volume delivered to the nares of the animal while under avertin anesthesia (Belser et al., 2013a). Mice inoculated by either route exhibited severe disease at inoculation doses $>10^3$ PFU, leading to >20% weight loss and >50% lethality at each dilution (Fig. 1A–B). The 50% lethal dose (LD_{50}) for both inoculation routes were <100 PFU, though the LD_{50} for AR inoculation (16 PFU) was 5-fold lower compared with IN inoculation (89 PFU) (Table 1); survival curves between mice receiving comparable levels of virus either AR and IN were not statistically different by the log-rank (Mantel–Cox) test. Gimje/08 virus replicated efficiently in the lungs of mice inoculated by either route, exhibiting comparable infectivity by AR (50% mouse infectious dose (MID_{50})=8.9 PFU) or IN (MID_{50}=15.8 PFU) inoculation. While viral titers in the lungs day 3 p.i. were higher following AR inoculation at doses of 5×10^4 – 5×10^1 PFU compared with IN (Fig. 1), these differences were not statistically significant by the Mann–Whitney test; viral titers in this tissue on day 6 p.i. from mice inoculated with 5×10^4 PFU were approximately 1×10^5 PFU/ml for both inoculation routes and not statistically different (data not shown). Gimje/08 virus replicated to comparable low titers in the nose on day 3 and 6 p.i. following either inoculation route, with no statistical differences observed between IN and AR delivery (Fig. 1C). In summary, the HPAI H5N1 virus maintained a lethal phenotype following inoculation by either route, though AR inoculation was modestly more infectious and lethal than IN inoculation.

Next, we inoculated mice AR or IN with A/Anhui/1/13 (Anhui/1), a LPAI H7N9 virus isolated from a fatal human case. In general, Anhui/1 virus maintained a lethal phenotype following either inoculation route, though mice inoculated with 5×10^3 PFU of virus IN exhibited severe morbidity and >50% mortality compared with mice inoculated AR with an equivalent inoculation dose, leading to a 3-fold lower LD_{50} for IN delivery (3.4×10^3 PFU) compared with AR (1.1×10^4 PFU) (Fig. 2A–B, Table 1); survival curves between mice receiving comparable levels of virus either AR and IN were not statistically different by the log-rank (Mantel–Cox) test. Anhui/1 virus exhibited high infectivity following IN or AR delivery; infectious doses were <10 PFU for both routes. At intermediate doses, mice inoculated IN possessed higher viral titers in the lung on day 3 p.i. compared with AR inoculated mice, but these differences were not statistically significant by the Mann–Whitney test. Similar to infection with the H5N1 virus, mice inoculated with Anhui/1 possessed day 6 p.i. viral titers in the lung that were not statistically different between IN

and AR routes, reaching titers >3×10^5 PFU/ml. In the nose, differences in viral titers were within 10-fold between both inoculation routes on day 3 and 6 p.i. (Fig. 2C). These results indicate that both routes were highly infectious and maintained a lethal phenotype for Anhui/1 in mice, though virus delivered IN was slightly more infectious and lethal than that delivered by the AR route.

A/Mexico/4482/09 (H1N1pdm09) virus (Mex/4482), isolated during the 2009 H1N1 pandemic from a human case with severe respiratory disease (Maines et al., 2009), replicated efficiently in mice in the absence of lethality following IN inoculation in a previous study (Belser et al., 2010). In accord with this finding, mice inoculated by either the IN or AR route exhibited only moderate morbidity (mean maximum weight loss approximately 10%) and no lethality (Fig. 3A). Mex/4482 virus was highly infectious by either inoculation route, with MID_{50} values of <2 PFU for both routes (Table 1). Viral titers in the lungs were slightly higher following IN inoculation compared with AR on day 3 p.i. but not statistically significant by the Mann–Whitney test; by day 6 p.i. titers were generally comparable by both inoculation routes (Fig. 3B). Virus was not consistently detected in the noses of mice by either inoculation route. In summary, both AR and IN inoculation routes yielded generally similar levels of infectivity, disease progression, and viral replication in mice challenged with a H1N1pdm09 virus.

The majority of comparative studies examining AR and IN inoculation in mice to date have used only one virus, making it difficult to understand the contribution of inherent viral virulence. Using both HPAI and LPAI viruses as well as a human-adapted pandemic virus in the present study has provided a more comprehensive assessment of the inherent variability among influenza viruses, and illustrates that subtle augmentation or diminishment of virus infectivity or viral titers between AR and IN inoculation routes in mice is likely attributable to strain-specific differences and not delivery methods, though future studies examining additional viruses and virus subtypes are needed to corroborate this observation. It is important to note that the use of 50 μl of liquid suspension for IN inoculations is a standard, reliable volume to achieve consistent murine infectivity and has been used often in similar comparative studies (Johansson and Kilbourne, 1991; Larson et al., 1976; Sherwood et al., 1988). However, the volume of virus inoculum administered IN can dramatically alter the resulting severity of disease in mice (Miller et al., 2013); studies which have demonstrated enhanced virulence in mice following inhalation versus intranasal inoculation have used a wide range of volumes (30–100 μl) for IN inoculation which may have contributed to these findings (Bowen et al., 2012; Smith et al., 2011). Comparable results between delivery methods were observed employing both egg-grown (H5N1, H7N9) and cell-grown (H1N1) virus preparations (Belser et al., 2013a, 2013b; Maines et al., 2009); while further studies are needed to closely examine the potential contribution of the growth matrix in the aerosolization of influenza viruses, our results support prior studies in mammalian models which indicate that the choice of propagation method for the starting virus stock does not adversely influence the resulting pathogenicity observed between intranasal or aerosol delivery (Gustin et al., 2013).

To date, comparison of virulence following AR or IN inoculation of HPAI viruses and other virus strains with pandemic potential has been largely restricted to the ferret model. These

studies have shown that inoculation with HPAI H5N1 viruses by both AR and IN inoculation routes are capable of causing a systemic and fatal disease in this species (Gustin et al., 2011, 2013; Lednicky et al., 2010). Ferrets inoculated AR with low doses of a HPAI H5N1 virus shed significantly higher nasal wash virus titers than ferrets inoculated IN, though this difference was abrogated when ferrets were challenged with higher viral doses (Gustin et al., 2011). While these studies using HPAI viruses in the ferret model have revealed inoculation method-specific differences in morbidity and kinetics of virus shedding in the upper respiratory tract (Gustin et al., 2013), comparable studies in the murine model were lacking. As such, our findings extend work in ferret model and highlight subtle strain-specific and subtype-specific differences in virulence between different inoculation routes in mice, while further underscoring the utility of both inoculation routes for pathogenesis studies.

Whole-body AR exposure systems offer several advantages to traditional IN inoculation. Notably, mice remain unanesthetized for the duration of the exposure, eliminating any confounding physiologic effects of anesthesia administration (Gargiulo et al., 2012). Nose-only AR methods typically require prior training of mice and/or the use of restraints, which can cause significant reductions in baseline breathing parameters of the animals (Rasid et al., 2012). While whole-body exposure does leave the fur of mice exposed to aerosolized virus, transmission of virus to naïve cage-mates was not observed (data not shown), in agreement with prior studies which indicate that residual virus on the fur of exposed mice is not transmissible (Schulman and Kilbourne, 1963). Mice remained alert and active for the duration of the exposure, with viral titers in AR inoculated mice exhibiting a comparable range of titers to those observed in IN inoculated mice with all viruses tested, indicating that huddling of mice during the exposure period leading to unequal inhaled doses did not occur (Bowen et al., 2012). The comprehensive examination of three virus strains in this study, yielding generally comparable results between all virus dilutions and parameters examined, demonstrates in triplicate the reproducibility of the methods used and results obtained between IN and AR delivery methods.

In conclusion, all influenza viruses tested were capable of mounting a productive respiratory infection in mice following AR inoculation, with generally comparable infectivity, disease progression, and lethality to that observed in IN inoculated mice. While potential strain-specific differences in virulence were observed, the magnitude of these differences were not statistically significant and were within the scope of previously published studies, suggesting that both inoculation routes are suitable for assessing influenza virus pathogenesis in this species. Further examination of inflammatory responses and cell-mediated immunity elicited following IN or AR virus challenge are warranted to determine the contribution of inoculation route on these immunological parameters (Johansson and Kilbourne, 1991; Rivers et al., 2013; Smith et al., 2011).

Acknowledgments

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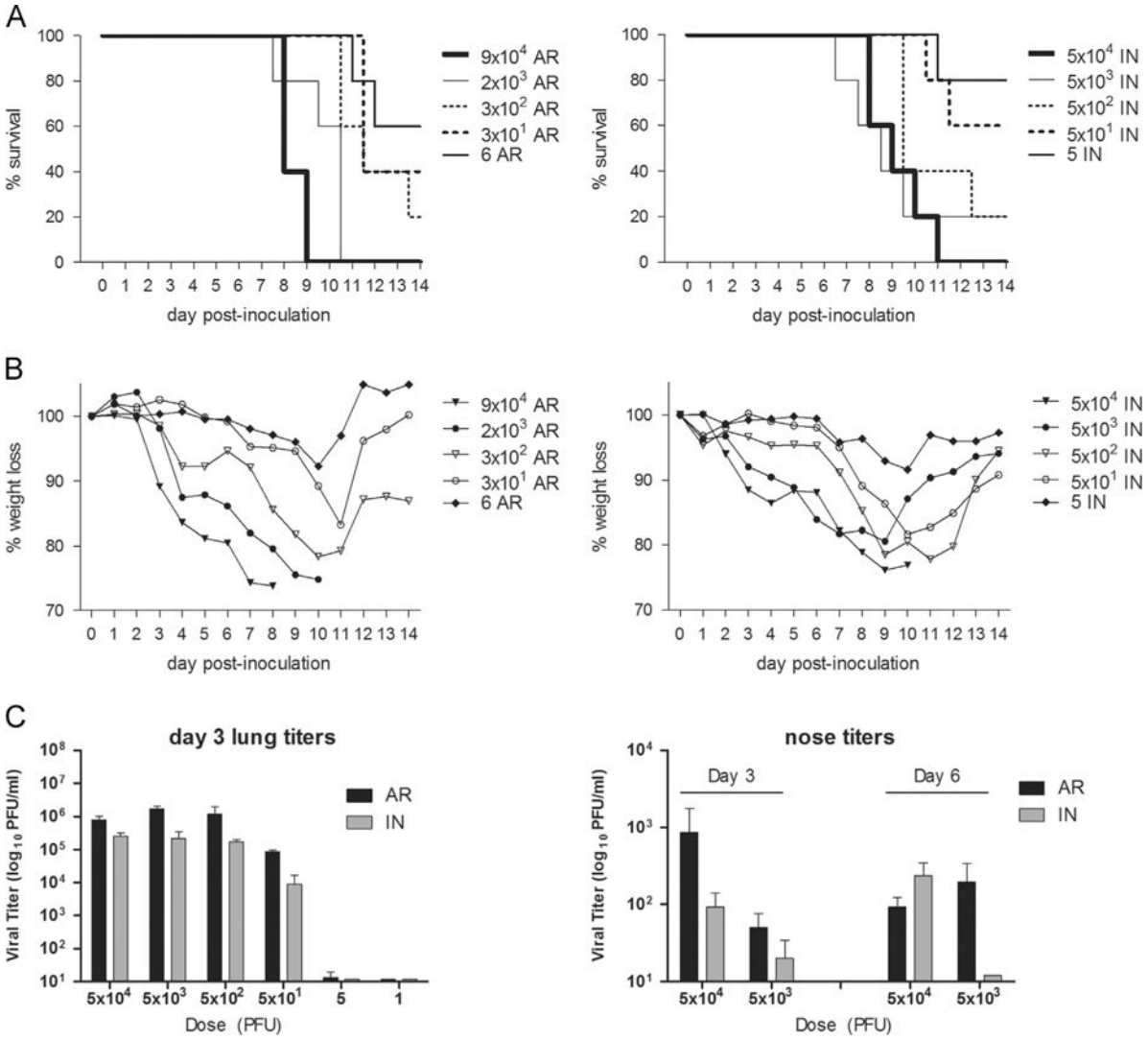

Fig. 1.

Comparison of mortality, morbidity, and viral titers following AR or IN inoculation of mice with HPAI H5N1 virus. Mice (5 per group) were inoculated by the AR or IN route with 10-fold serial dilutions of A/ck/Korea/Gimje/08 virus and monitored daily for 14 days for survival (A) and weight loss (B). Any mouse which lost >25% initial body weight was euthanized. Lungs and noses were collected from an additional 3 mice per group on day 3 p.i. for virus titration (C). Tissues were titered in MDCK cells by standard plaque assay and reported as $\log_{10}$ PFU/ml. The limit of detection was 10 PFU. Viral challenge for each group is presented in the legend (A and B) or *x* axis (C) as PFU.

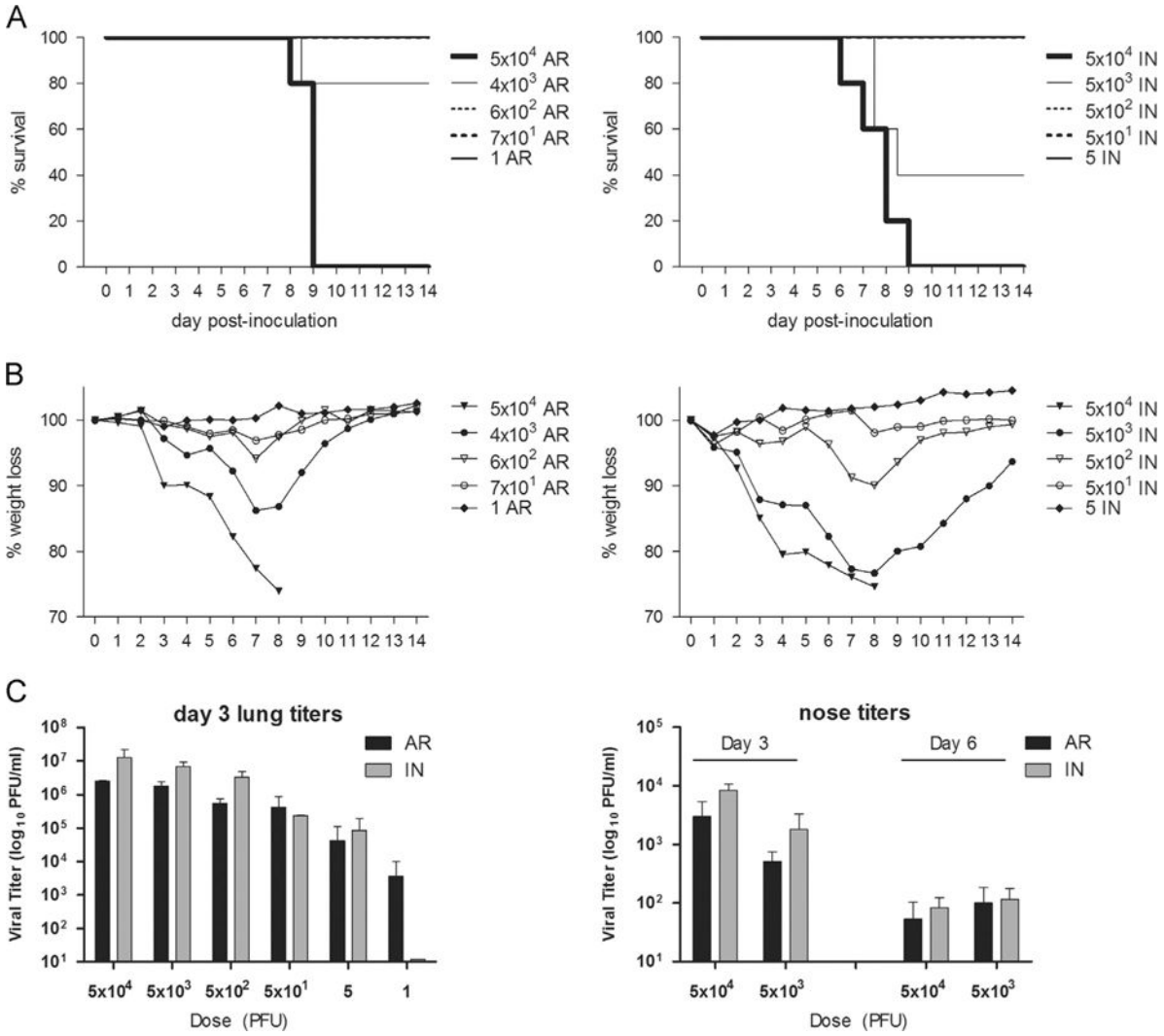

Fig. 2.

Comparison of mortality, morbidity, and viral titers following AR or IN inoculation of mice with LPAI H7N9 virus. Mice (5 per group) were inoculated by the AR or IN route with 10-fold serial dilutions of A/Anhui/1/13 virus and monitored daily for 14 days for survival (A) and weight loss (B). Any mouse which lost >25% initial body weight was euthanized. Lungs and noses were collected from an additional 3 mice per group on day 3 p.i. for virus titration (C). Tissues were titered in MDCK cells by standard plaque assay and reported as $\log_{10}$ PFU/ml. The limit of detection was 10 PFU. Viral challenge for each group is presented in the legend (A and B) or *x* axis (C) as PFU.

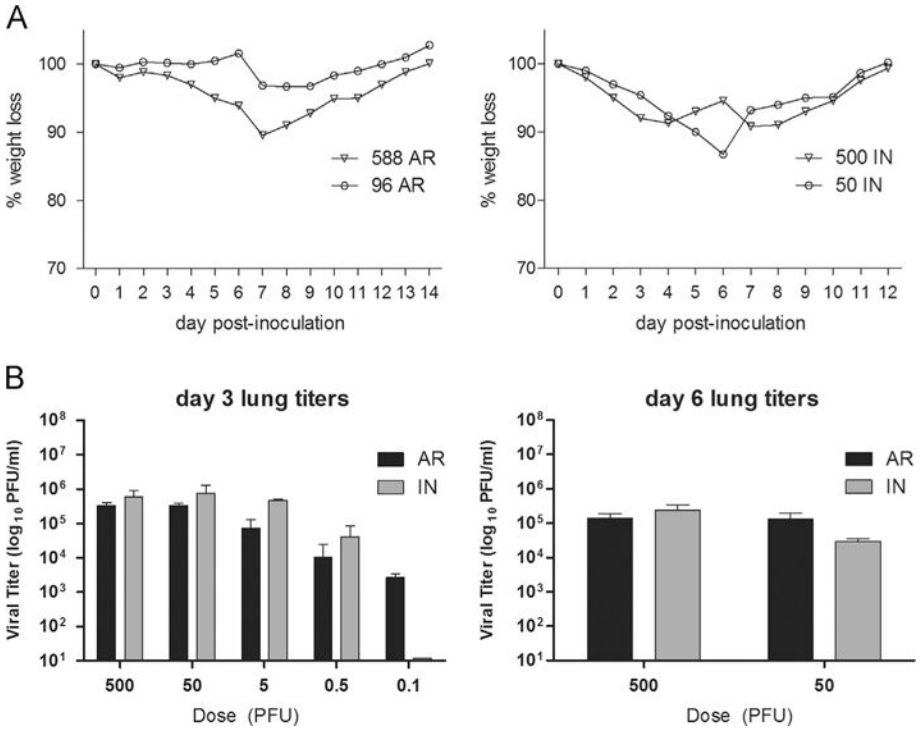

Fig. 3.

Comparison of mortality, morbidity, and viral titers following AR or IN inoculation of mice with 2009 H1N1 virus. Mice (5 per group) were inoculated by the AR or IN route with 10-fold serial dilutions of A/Mexico/4482/09 virus and monitored daily for 14 days for weight loss (A). Lungs were collected from an additional 3 mice per group on days 3 and 6 p.i. for virus titration (B). Tissues were titered in MDCK cells by standard plaque assay and reported as $\log_{10}$ PFU/ml. The limit of detection was 10 PFU. Viral challenge for each group is presented in the legend (A) or *x* axis (B) as PFU.

Table 1

Infectivity and lethality of influenza virus following AR or IN inoculation.

Virus[a]	Subtype	Infectivity (MID_{50})[b]		Lethality (LD_{50})[b]	
		AR	IN	AR	IN
A/ck/Korea/Gimje/08	HPAI H5N1	8.9	15.8	15.8	88.9
A/Anhui/1/13	LPAI H7N9	8.9	1.6	1.1×10^4	3.4×10^3
A/Mexico/4482/09	2009 H1N1	0.3	1.5	NA[c]	NA

[a] Virus stock propagations are described previously in (Belser et al., 2013a, 2013b; Maines et al., 2009), with all experiments conducted in biosafety level 3 containment (Chosewood and Wilson, 2009).

[b] Values are expressed as the PFU required to give 1 MID_{50} or 1 LD_{50}, respectively.

[c] NA, not applicable as all mice survived the virus challenge.